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Rapid Laser Flash Photoaffinity Labeling of Binding Sites for a Noncompetitive Inhibitor of the Acetylcholine Receptor†

Peter Muhn, Alfred Fahr, and Ferdinand Hucho*

ABSTRACT: Photoaffinity labeling of the nicotinic acetylcholine receptor from *Torpedo marmorata* electric tissue was performed in the presence of cholinergic effectors in the millisecond to second time range by a combination of a stopped-flow apparatus and a high-energy pulse laser. The label applied was [^{3}H]triphenylmethylphosphonium, a lipophilic cation previously shown to be a specific blocker of the acetylcholine receptor ion channel. With the receptor in the resting state most of the label was incorporated into the α polypeptide chains. In the presence of agonists and antagonists increasing incorporation into the δ- and (less pronounced) the β-chain was observed. The time course of this increase had a half-life of about 0.4 s, being slower than receptor activation and channel opening. In the resting, active, and even rapidly desensitized state, the α polypeptide chains appear to be the primary targets of the photoaffinity reaction. The action spectrum of the photolabeling has a sharp maximum at λ = 270 nm and a small-side maximum at λ = 290 nm. It does not resemble the absorption spectrum of the label and may hint at amino acid side chains as the moieties activated by UV light causing the photolabeling. The effector specificity of the observed slow increase of label incorporation into the δ polypeptide chain was investigated. It does not prove that slow desensitization is the underlying event. The agonists acetylcholine and carbamoylcholine as well as treatment of receptor-rich membranes with phospholipase A_2 (but not phospholipase D) triggered labeling of δ, but antagonists such as D-tubocurarine and most conspicuously flaxedil had a similar effect.

The nicotinic acetylcholine receptor (AChR)[1] is present in the postsynaptic membrane in at least three different functional states, termed resting (with its ion channel closed), active (ion channel open), and inactive or desensitized [ion channel closed, affinity for agonists 2 orders of magnitude higher than in the resting state [for a recent review, see Changeux (1981)]]. The molecular mechanism of the transitions between these states is generally grossly described as conformational changes of the receptor protein, but no details are available as to the subunits or the part of the primary structure involved. It is well established now that the α polypeptide chains (M_r 40 000) contain the binding site for agonists and antagonists. But there is little information concerning the role of the other three polypeptide chains of the receptor complex (β, M_r 48 000; γ, M_r 60 000; δ, M_r 68 000). The δ-chain has been implied in high-affinity binding of noncompetitive blockers (NCBs) (Oswald & Changeux, 1981). NCBs are allosteric inhibitors of the AChR and its ion channel but at least some of them appear to be direct steric blockers of the channel as well (Heidmann et al., 1983). For elucidation of the blocking mechanisms of NCBs—the allosteric and the steric one—and for identification of their site of action, the following observations could be helpful: Several NCBs have been shown to react covalently with AChR upon UV irradiation of the receptor–NCB complex (Oswald & Changeux, 1981). For example, the lipophilic cation [^{3}H]triphenylmethylphosphonium ([^{3}H]TPMP$^+$) has been shown to block reversibly the cation flux through the ion channel (Lauffer & Hucho, 1982) and to react during UV irradiation covalently with various receptor subunits depending on the receptor state (Muhn & Hucho, 1983). In the absence of cholinergic ef-

† From the Freie Universität Berlin, Institut für Biochemie and Institut für Atom- und Festkörperphysik, Abteilung Biophysik, 1000 Berlin 33, West Germany. *Received November 18, 1983.* This work was supported by the Land Berlin, FGS Biomembranen, the Deutsche Forschungsgemeinschaft, and the Fonds der Chemischen Industrie.

[1] Abbreviations: [^{3}H]TPMP$^+$, [^{3}H]triphenylmethylphosphonium; AChR, nicotinic acetylcholine receptor; NCB, noncompetitive blocker; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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fectors it is mainly the α-chain and in the presence of agonists and certain antagonists at equilibrium it is the δ-chain that incorporates the label. This indicates that these effectors have a 2-fold effect: they stimulate binding and by that the covalent reaction of [^{3}H]TPMP$^+$ and they cause a shift of the primary reaction site from the α to the δ polypeptide chain.

The UV-induced covalent reaction of compounds like [^{3}H]TPMP$^+$ therefore can be used to monitor structural changes during AChR activity and to label the molecular moieties involved. A prerequisite for this type of experiment is of course a time scale of the photolabeling comparable to the time scale of the physiological events, e.g., AChR activation or desensitization. Here we propose a combination of the stopped-flow technique with a pulse laser for labeling AChR with [^{3}H]TPMP$^+$ in the time range of milliseconds to seconds after formation of the receptor–agonist complex.

Materials and Methods

Reagents. α-Bungarotoxin was from Boehringer, Mannheim, and D-tubocurarine and TPMP$^+$ bromide were obtained from Sigma. [^{3}H]TPMP$^+$ iodide (specific radioactivity 57.4 Ci/mmol) was purchased from New England Nuclear; for use the reagent was evaporated to dryness and redissolved in a 3-fold volume of N_2-deaereated Ringer's solution. The final concentration was now 330 000 cpm/μL. All other reagents were of the highest purity commercially available.

Preparation of Membrane Fragments from Torpedo Electric Tissue. Microsacs from *Torpedo marmorata* were prepared as described (Schiebler & Hucho, 1978) and recovered from sucrose gradients by dilution in H_2O (5-fold) and sedimentation (35000g/90 min). Membrane fragments were then resuspended in sodium Ringer's solution and left for equilibration overnight in ice. Protein was determined according to Lowry et al. (1951). Vesicles were then diluted to a protein concentration of 1.00 mg/mL with N_2-deaereated sodium Ringer's solution. Specific [^{125}I]-α-bungarotoxin binding activity was 1500–2500 nmol/g of protein.

Rapid Photoaffinity Labeling of Receptor-Rich Membranes with [^{3}H]TPMP$^+$. (1) Laser Equipment. We used a Q-switched Nd:YAG DCR-2A laser (Quanta-Ray) at a wavelength of 266 nm (4-fold the primary frequency) in a single-pulse mode. The pulse energy was typically 15 mJ and pulse duration 4–5 ns. The beam was focused by a 300-mm lens and the stopped-flow apparatus was positioned at a distance that allowed full irradiation of the reaction chamber. The laser flash was triggered by a self-built variable-delay pulse generator, which in turn was triggered by the stop switch of the stopped-flow apparatus.

(2) Stopped-Flow Apparatus. A commercial piece of equipment (Sigma Instrumente GmBH, Berlin) was modified for our purpose. A thermoresistant mixing chamber was built from German silver. The reaction chamber had a diameter of 2.4 mm, a beam path of 5 mm, and a total volume of 22 μL. The experimentally determined dead time was 2.4 ms at 3.2 bars of pressure on the pistons. Complete removal of the irradiated sample was attained by an optimized (avoidance of air bubbling) tubing connected to the outlet of the mixing chamber and a Hamilton air-locked syringe. The total dead volume was 8 μL.

Before being rapidly mixed in the stopped-flow apparatus membranes were either preincubated with 10^{-4} M carbamoylcholine or 0.5 μM [^{3}H]TPMP$^+$ or left in the native state. Final concentrations of proteins and effectors were the same as described for the action spectrum experiment (see below).

Slow Photoaffinity Labeling of Receptor-Rich Membranes with [^{3}H]TPMP$^+$. Essentially the procedure described (Muhn & Hucho, 1983) was applied. In the case of equilibrium experiments the samples irradiated had a 36-μL total volume (18 μL of membranes, 1 mg/mL protein, and 12 μL of 3×10^{-4} M cholinergic effector, unless otherwise indicated in the text), 6 μL (=2 μCi) of [^{3}H]TPMP$^+$ (final concentration 1 μM), in sodium Ringer's solution. The equilibration time was 5 min at room temperature.

Action Spectrum of Photoaffinity Labeling. Twenty-eight microliters of *T. marmorata* microsacs (1 mg of protein/mL), 7.5 μL of [^{3}H]TPMP$^+$ (0.33 μCi/μL), and 15 μL of 3×10^{-4} M carbamoylcholine were incubated for 5 min at room temperature, then placed into a 1-mL quartz cuvette, and positioned into a Zeiss MQ 4 photometer so that the whole sample was irradiated by the light beam. The wavelength of a 450-W xenon high-pressure lamp was adjusted with the monochromator. Each sample was irradiated for 5 min, then removed, and analyzed.

The samples had a total volume of 50.5 μL with the same concentrations as above. The radioactivity incorporated was determined by DEAE filter binding (Millipore) of the irradiated samples and with 1% Triton X-100 (Serva) solubilized membranes. The filters were washed 3 times with 30 mL of buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.1% Triton X-100), and the bound activity was measured in Supertron liquid scintillation cocktail in a liquid scintillation counter.

SDS–Polyacrylamide Gel Electrophoresis. Thirty microliters of irradiated samples was mixed with 30 μL of Laemmli sample buffer of double concentration, left at room temperature for 30 min, and then subjected to SDS gel electrophoresis with a 3% acrylamide upper and a 10% acrylamide lower gel (Laemmli, 1970). Gels were fixed, stained, destained, and fluorographed as described before (Muhn & Hucho, 1983).

Quantitative determination of incorporated radioactivity was attained by cutting out the respective bands of the Coomassie blue stained gel, solubilizing in Toluol/Omnifluor (4 g/L)/Protosol (3%) (10 mL/band, 18 h at room temperature), and counting in a liquid scintillation counter.

Effects of phospholipases on the covalent labeling of AChR membranes with [^{3}H]TPMP$^+$ were determined by preincubating receptor-rich membranes with increasing amounts of commercial phospholipases A_2 from *Crotalus durissus terrificus* (samples from Boehringer and Sigma were used and gave the same results) and phospholipase D (Boehringer). Between 0.2 and 2×10^{-4} units were incubated with receptor-rich membranes for 1 h at room temperature in a final volume of 18 μL and 1 mg/mL protein. Subsequently the samples were incubated for further 5 min at room temperature with [^{3}H]TPMP$^+$ (2 μCi) and finally irradiated with UV light as described.

Results

Action Spectrum of the Photoreaction. In previous experiments we had observed that there is a significant difference in the photolabeling patterns obtained with AChR from different species of electric fish. The observed incorporation of [^{3}H]TPMP$^+$ into the δ polypeptide chain of the receptor complex stimulated by carbamoylcholine was reproducibly obtained with receptor from *T. marmorata* but not with preparations from *Torpedo californica*. This indicated that it was not truly the label ([^{3}H]TPMP$^+$) that was activated by light but some components of the protein. This component then would be absent or not active in AChR from *T. californica*.

To investigate this possibility, we performed the photolabeling with UV light of varying wavelength. Figure 1 shows

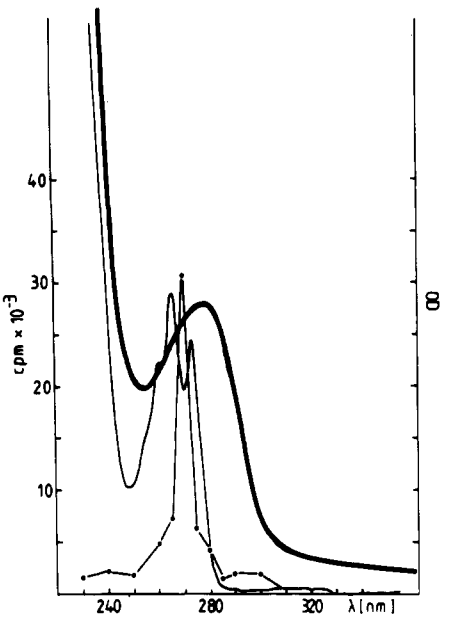

FIGURE 1: Action spectrum of the photoreaction: (heavy line) absorption spectrum of AChR (1 mg/mL, dissolved in 4% cholate–100 mM phosphate buffer, pH 7); (light line) absorption spectrum of $TPMP^+$ (10^{-4} M in H_2O); (●) radioactivity bound to AChR.

that light of $\lambda = 270$ nm was most efficient in this reaction. Comparison of the action spectrum with the absorption spectra of $TPMP^+$ and AChR, respectively, shows no similarity with the $TPMP^+$ spectrum. Maximum efficiency is obtained close to the maximum of the protein spectrum. This observation and the small-side maximum around 290 nm may hint at the involvement of aromatic amino acid side chains in the photoreaction.

Irradiation of $[^3H]TPMP^+$ did not yield a long-lived reaction product. After the light source was turned off, no further incorporation of label was observed.

Photolabeling by Laser Light Pulses. Previously we have shown that single high-energy light pulses generated by an appropriate laser are sufficient to trigger the photoreaction between $[^3H]TPMP^+$ and AChR (Muhn et al., 1983). We combined a pulse laser with a stopped-flow apparatus, allowing the photoreaction to start at various time lapses after mixing AChR with effectors. The reaction products after irradiation with laser light of 266 nm were subsequently removed from the stopped-flow mixing chamber and analyzed by SDS–polyacrylamide gel electrophoresis. A typical experiment is shown in Figure 2. Figure 2a shows that radioactivity incorporated into the α polypeptide chain of the receptor is not stimulated by an agonist within several hundred milliseconds after mixing the AChR–$[^3H]TPMP^+$ complex with carbamoylcholine. Labeling of α-chains therefore seems to be similar in the resting, activated, and desensitized receptor. On the other hand, a steady increase with time is observed with the δ polypeptide chain (Figure 2b). The agonist carbamoylcholine stimulated incorporation of radioactivity into the δ-chain by laser pulse irradiation with a half-time of about 0.4 s.

We do not know whether this half-time is due to a conformational change within the protein resulting in a state with a higher affinity and/or reactivity of the δ polypeptide chain toward $[^3H]TPMP^+$. It could as well represent slow $[^3H]$-$TPMP^+$ binding after a rapid change in the protein conformation. Figure 2c shows that binding to another state of the receptor reached after equilibration with carbamoylcholine is slow (half-time 4 s). But this equilibrium is very different from the transient states obtained shortly after mixing AChR with agonist. At any rate, identical labeling kinetics are obtained when AChR is preequilibrated with $[^3H]TPMP^+$ or when $[^3H]TPMP^+$ is added to the receptor together with the agonist.

Dose–Response Curve of Agonist-Stimulated Photolabeling. We attempted to obtain further information concerning the functional correlate underlying the observed conformational change resulting in the altered labeling pattern of the receptor polypeptide chains. It has been observed before that the carbamoylcholine concentration causing half-maximal desensitization is in the micromolar range while half-maximal activation is obtained with about 10 μM carbamoylcholine (Oswald et al., 1983). Figure 3 shows a dose–response curve of the carbamoylcholine- and the acetylcholine-stimulated $[^3H]TPMP^+$ labeling of the δ-chain. Labeling was performed in this case not with laser flashes and the rapid mixing technique but with equilibrated AChR–agonist complexes and irradiation with a normal UV lamp. Half-maximal response is obtained at concentrations causing desensitization but not

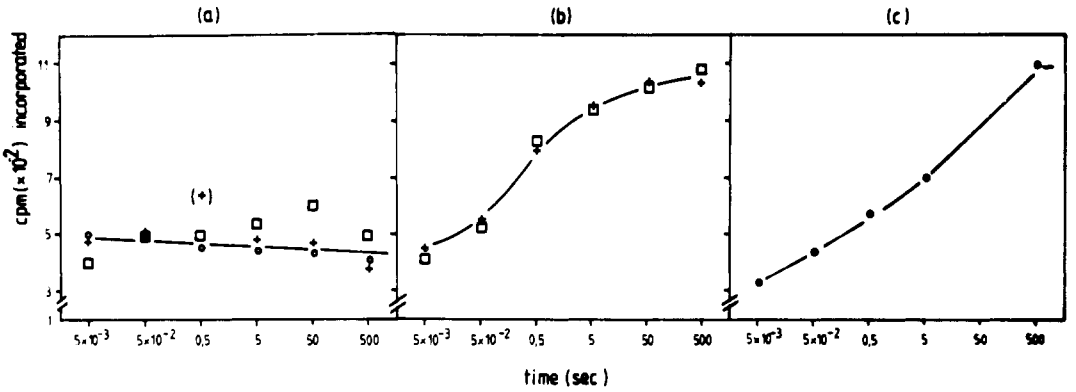

FIGURE 2: Rapid photoaffinity labeling of receptor-rich membranes: stopped-flow experiment. Receptor-rich membranes (1 mg/mL) were rapidly mixed (see Materials and Methods) with $[^3H]TPMP^+$ and carbamoylcholine and irradiated with one high-energy laser pulse at different time intervals after mixing in a stopped-flow apparatus. Irradiated samples were analyzed by electrophoresis on a 10% acrylamide gel (Laemmli, 1970). α and δ polypeptide chains were cut out of the Coomassie blue stained gel, and the covalently bound radioactivity was determined as described under Materials and Methods. (a) Time course of incorporation of radioactivity in the α polypeptide chains: (+) AChR-rich membranes rapidly mixed with 2×10^{-4} M carbamoylcholine and $[^3H]TPMP^+$; (□) AChR-rich membranes preincubated with $[^3H]TPMP^+$ and then rapidly mixed with 2×10^{-4} M carbamoylcholine; (○) AChR-rich membranes preincubated for 15 min with 10^{-4} M carbamoylcholine, then rapidly mixed with $[^3H]TPMP^+$, and irradiated with one laser pulse ($\lambda = 266$ nm). (b) Time course of incorporation of radioactivity in the δ polypeptide chain: for experimental conditions and symbols see (a). (c) Time course of incorporation of radioactivity in the δ polypeptide chain: AChR preequilibrated with 10^{-4} M carbamoylcholine (15 min, room temperature) and then rapidly mixed with 10^{-4} M carbamoylcholine and 0.5 μM $[^3H]TPMP^+$.

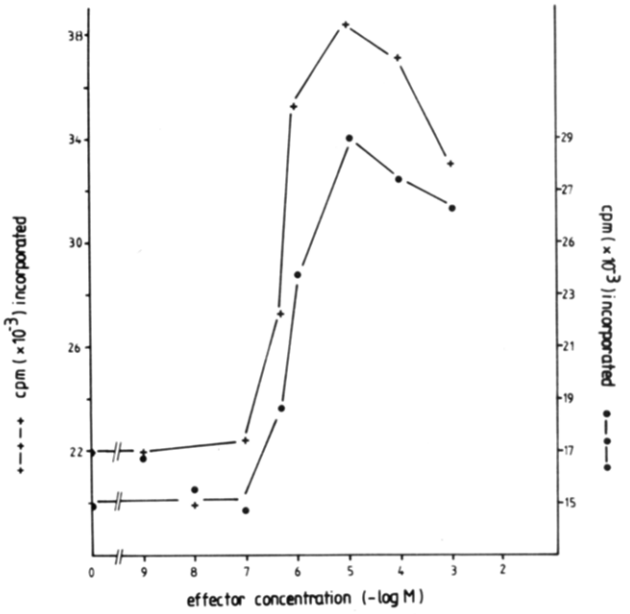

FIGURE 3: Dose dependence of photoaffinity labeling of the δ polypeptide chain stimulated by agonists. Receptor-rich membranes (1 mg/mL) were incubated with 10^{-4} M eserine and different concentrations of either acetylcholine (+) or carbamoylcholine (●) and 1 μM [^{3}H]TPMP$^+$ for 5 min at room temperature before being irradiated with a Hg lamp for 3 min at 4 °C [see Materials and Methods and Muhn & Hucho (1983)]. After electrophoresis and Coomassie blue staining δ-subunits were cut out and covalently incorporated radioactivity was analyzed as described under Materials and Methods.

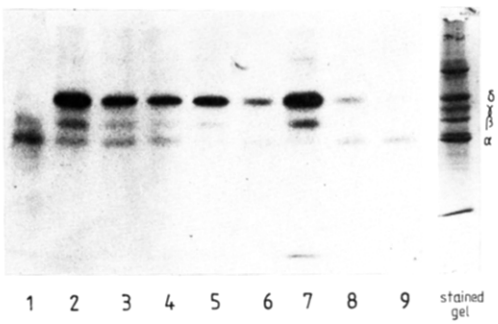

FIGURE 4: Influence of different cholinergic effectors on the photoaffinity labeling of AChR with [^{3}H]TPMP$^+$. Receptor-rich membranes (1 mg/mL) were incubated with various effectors (10^{-4} M) and with 1 μM [^{3}H]TPMP$^+$ for 5 min at room temperature. Subsequently, the mixture was irradiated for 2 min at 4 °C. Samples were electrophoresed; for fluorography the gel was fixed in 90% 2-propanol/10% acetic acid, soaked in 1 M sodium salicylate for 1 h, dried, and exposed for 5 days to Kodak X-O-Mat AR film. Effectors used were (10^{-4} M each) (1) none (only sodium Ringer's solution), (2) carbamoylcholine, (3) hexamethonium, (4) decamethonium, (5) D-tubocurarine, (6) flaxedil, (7) α-bungarotoxin (10^{-5} M) (commercial sample containing proteases and lipases; see the text and Figure 5), (8) procaine, (9) tetracaine, and for reference the band pattern of the Coomassie blue stained SDS–polyacrylamide gel of receptor membranes.

activation. The dose–response curve correlates well with the receptor occupancy at various agonist concentrations. Note the decrease of labeling at high agonist concentrations. It may be due to a weak competition of carbamoylcholine for the NCB site.

Effector Specificity of Stimulated Photolabeling. The slow time course and arguments forwarded by other authors (see Discussion) may suggest that it is actually the desensitized state that is labeled by [^{3}H]TPMP$^+$ after equilibration of the receptor with ligands. Figure 4 shows the labeling pattern obtained by UV irradiation in the presence of various cholinergic ligands. Track 1 shows an SDS–polyacrylamide gel of AChR photolabeled in the resting state (i.e., in the absence of any ligand). Mainly the α-chain is labeled. Track 2 shows that stimulation by carbamoylcholine causes labeling of mainly the δ-chain (and to a smaller extent the β-chain). Track 6 shows that a qualitatively similar labeling pattern is obtained with the antagonist flaxedil. This confirms our previous finding (Muhn & Hucho, 1983) that antagonists, e.g., D-tubocurarine and hexamethonium and now flaxedil, stimulate labeling of the δ-chain similarly as agonists. Finally in tracks 8 and 9 it is obvious that the local anesthetics procaine and tetracaine, noncompetitive blockers, inhibit labeling.

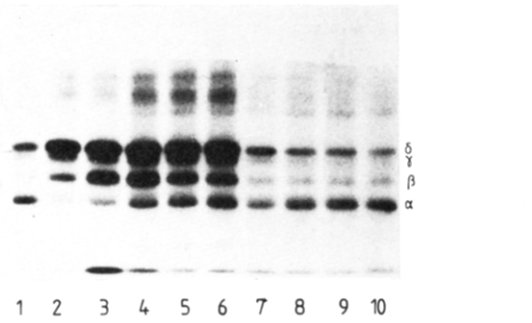

FIGURE 5: Effects of phospholipases A and D on the covalent [^{3}H]TPMP$^+$-labeling pattern. Receptor-rich membranes (1 mg/mL, in sodium Ringer's solution) were preincubated with different amounts of lipases for 1 h at room temperature. 18-μL samples were then incubated for 5 min at room temperature with 1 μM [^{3}H]TPMP$^+$ and irradiated for 2 min. Fluorography of the electrophoresis gels of the samples shows (track 1) native membranes (without effector), (track 2) photolabeling in the presence of 10^{-4} M carbamoylcholine, (tracks 3–6) photolabeling of phospholipase A_2 treated membranes [(track 3) 2×10^{-1} unit of lipase/18 μL of sample; (track 4) 2×10^{-2} unit/18 μL; (track 5) 2×10^{-3} unit/18 μL; (track 6) 2×10^{-4} unit/18 μL], and (tracks 7–10) photolabeling of phospholipase D treated membranes [(track 7) 2×10^{-1} unit of the lipase/18 μL of sample; (track 8) 2×10^{-2} unit/18 μL; (track 9) 2×10^{-3} unit/18 μL; (track 10) 2×10^{-4} unit/18 μL].

In a previous publication (Muhn & Hucho, 1983) we had presented evidence that even the strong competitive antagonist α-bungarotoxin stimulated photolabeling of the δ-chain resembling in this respect the other agonists and antagonists [but see Added in Proof of Muhn & Hucho (1983)]. In Figure 4, track 7, this effect was reproduced. Part of this effect may have been due to proteolytic contamination in the commercial toxin sample, and most of the effect probably was caused by a lipase present in this toxin. α-Bungarotoxin purified in our laboratory showed little if any stimulatory effect. On the contrary, it even prevented labeling of the α-chain. Figure 5 shows the labeling patterns obtained by UV irradiation of a receptor–[^{3}H]TPMP$^+$ mixture after treatment with phospholipase A (left) and phospholipase D (right). Two commercial preparations of α-bungarotoxin contained considerable phospholipase A activity as determined by thin-layer chromatography of the degradation products resulting from incubating phosphatidylcholine with both toxin samples. Phospholipase A treatment strongly stimulates labeling of the β- and especially the δ-chain. Phospholipase D has virtually no effect.

Discussion

The following conclusions can be drawn from these experiments. (1) The noncompetitive blocker [^{3}H]TPMP$^+$ is a photoaffinity label of the α polypeptide chain of the AChR in its resting state. This chain appears to contain the binding site for this NCB. (2) With AChR from *T. marmorata* agonists and antagonists shift the receptor to a state in which

the δ-chain is the main target of the photolabel. This state is unlikely to be the desensitized state since it would not be stabilized by, e.g., flaxedil and would be reached with another time constant.

It has been proposed that certain NCBs (phencyclidine, histrionicotoxin) bind to a single high-affinity site located in the central cavity formed by the five polypeptide chains of the receptor complex (Oswald et al., 1983). At this location they might block the ion channel directly (sterically) and indirectly by stimulating the transition to the desensitized state (allosterically). [^{3}H]TPMP$^+$ probably binds to the same site as phencyclidine, a proven noncompetitive blocker of the AChR ion channel (Eldefrawi et al., 1980), because it competes with [^{3}H]phencyclidine for a common binding site, and as for phencyclidine and histrionicotoxin there is only one high-affinity binding site per receptor molecule (Lauffer, 1983). During UV irradiation [^{3}H]TPMP$^+$ reacts with the α-chain, when the receptor is in its resting state and also after activation by agonists. This makes it unlikely that the [^{3}H]TPMP$^+$ binding site is located in the central cavity in touch with all five polypeptide chains. Of course, we cannot exclude that the lack of photolabeling of the β-, γ-, and δ-chains in the resting state is due to a lack of reactivity of these proteins toward [^{3}H]TPMP$^+$. But this appears unlikely; [^{3}H]-H_{12}-histrionicotoxin, [^{3}H]phencyclidine, and [^{3}H]trimethisoquin also did not label all five polypeptide chains during UV irradiation (Oswald & Changeux, 1981). They showed a significant preference for the δ-chain labeling, which was stimulated by agonists and certain antagonists. [^{3}H]Chlorpromazine was less selective in this respect and indicates that all polypeptide chains can undergo photoreactions.

NCBs bind with low affinity also to the agonist binding site, located on the α-chain as well. Binding of [^{3}H]TPMP$^+$ to this site becomes significant only at much higher concentrations (millimolar; Lauffer, 1982; Lauffer & Hucho, 1982) than the ones used in our photolabeling experiments (micromolar). Furthermore, [^{3}H]TPMP$^+$ is displaced from its binding site on the α-chain by local anesthetics like tetracaine (Figure 4). Therefore, we think it is not the agonist binding site but a NCB binding site that we photoaffinity label on the α-chain of AChR in its resting state.

It is furthermore noteworthy that very little radioactivity is incorporated during UV irradiation into the lipid phase. This is in contrast to most other NCBs used so far, which bind in addition to the single high-affinity NCB binding site to several low-affinity sites (Heidmann et al., 1983). These are presumed to be located at the lipid/protein interface or even in the lipid bilayer.

In the resting as well as in the activated (channel open) state photolabeling with [^{3}H]TPMP$^+$ occurs mainly on the α-chain. This poses the question whether the observed block of ion flux through the receptor channel correlates with the binding of the blocker to the α-chain. We cannot perform flux measurements in the millisecond time range (Hess et al., 1979; Neubig & Cohen, 1980), but since no ion flux at all can be stimulated by carbamoylcholine in the presence of TPMP$^+$ (Lauffer & Hucho, 1982), when both ligands are added simultaneously, one may conclude that both TPMP$^+$ binding and ion flux inhibition occur at the same site. These conclusions are based on the assumption that the reaction site for the covalent label is the same as the blocking site for the reversible ligand [^{3}H]TPMP$^+$. Our evidence for the validity of this is only indirect: the covalent labeling can be prevented by excess (100-fold) cold TPMP$^+$. Its pharmacology parallels that of the reversible blocking effect (it is prevented, e.g., by aminated local anaesthetics and stimulated by agonists and competitive antagonists), and it shows the same concentration dependence and saturation with respect to carbamoylcholine stimulation. Therefore, we conclude that TPMP$^+$ appears to block ion translocation by steric inhibition through binding to the α-polypeptide chain. This chain comprises at least an important part of the ion channel.

The meaning of the structural transition causing the shift of the site of photolabeling from the α to the δ (and β) polypeptide chain after stimulation with cholinergic effectors remains unclear. The stimulation of reversible binding and of irreversible labeling of the δ-chain with a photosensitive derivative of trimethisoquin has been proposed to represent AChR desensitization (Oswald & Changeux, 1981). At least in the case of [^{3}H]TPMP$^+$ we do not agree with this interpretation.

The dose–response curve for the agonist-stimulated covalent incorporation of the photoaffinity label (Figure 3) shows half-maximal response at concentratons causing desensitization but not activation. The latter requires 200-fold higher concentrations than the former (Neubig et al., 1982; Hess et al., 1979). This can be taken as evidence that the increase in photoaffinity labeling observed in the presence of cholinergic ligands is not due to receptor activation. But we do not think that it is due to receptor desensitization (slow desensitization): The time course of the stimulated labeling of the δ-chain is slower than the so-called intermediated transition (occurring in the 10–100-ms time range) (Heidmann & Changeux, 1979), the transition from the R to the I state of the four-state model proposed for the AChR (Neubig & Cohen, 1980), which is possibly related to "rapid desensitization" (Changeux, 1981). But it is also different from the slow desensitization: different in its time scale ($t_{1/2}$ = 0.4 s for the stimulation of the photolabeling vs. $t_{1/2}$ of seconds to minutes for the desensitization) and different in its effector specificity. The competitive antagonist flaxedil does not stimulate desensitization but enhances [^{3}H]TPMP$^+$ binding and photoaffinity labeling of the δ-chain. Perhaps this stimulation monitors a new conformational state. Its functional significance (if any) remains to be elucidated.

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Activation of Acetylcholinesterase by Monovalent (Na^+, K^+) and Divalent (Ca^{2+}, Mg^{2+}) Cations†

Peter Hofer, Urs Peter Fringeli,* and Wolfgang Heinrich Hopff

ABSTRACT: The activation of acetylcholinesterase [EC 3.1.1.7 (AChE)] by monovalent and divalent metal ions has been investigated by kinetic experiments under steady-state conditions (pH-stat method). It has been shown that at low ionic strength the enhancement of the activity by both monovalent and divalent metal ions can be explained as an electrostatic effect. Thereby, enhancement of the concentration of monovalent metal ions (Na^+ and K^+) acts by reducing the penetration depth of the electric field of carboxylate groups located at the active center whereby divalent metal ions (Ca^{2+} and Mg^{2+}) act by a complex formation with these charged groups.

It has been known for many years that the enzymatic activity of acetylcholinesterase [EC 3.1.1.7 (AChE)][1] is enhanced by alkaline metal ions (Na^+ and K^+) and alkaline-earth metal ions (Ca^{2+} and Mg^{2+}) whereby the activation caused by the second group is much greater than that caused by the first group (Nachmansohn, 1940). There are at least three explanations for this activating effect: (1) ionic strength effects according to the theories of Brönsted–Bjerrum and Debye–Hückel; (2) conformational changes as a result of a peripheral site occupation by the metal ions; (3) specific binding of the metal ion to the anionic subsite of the active centre.

Roufogalis & Wickson (1973, 1975) have shown that the activation by calcium ions is inhibited by a treatment of the enzyme with carboxyl group reagents such as carbodiimide. They interpreted this observation as the inhibition of an allosteric effect.

Nolte et al. (1980) explained the influence of the sodium concentration on the association rate constant of the cationic ligand *N*-methylacridinium with AChE as an ionic strength effect, which can be described by the Brönsted–Debye–Hückel theory.

Smissaert (1981) interpreted his results as a "specific salt effect". Thereby, the association of the Na^+ ions with the anionic subsite of the catalytic center reduces the reactivity (k_{cat}/K_m) of the enzyme.

Tomlinson et al. (1981) explained the activating effect of the divalent metal ions Ca^{2+} and Mg^{2+} as a conformational change which is induced by the occupation of a peripheral site of the enzyme by these ions. A voluminous literature deals with "peripheral anionic sites" of AChE. For a review the reader is referred to Rosenberry (1975), Bolger & Taylor (1979), and Berman et al. (1981).

In this work we want to examine whether the activation of AChE by mono- and divalent metal ions can be explained only by ionic strength effects or whether additional mechanisms have to be taken into consideration.

Materials and Methods

Enzyme Purification. AChE from the electric organ of *Torpedo marmorata* was purified by affinity chromatography as described by Hopff (1976). Density gradient centrifugation revealed that the preparation contained 50% of the globular 11S form and 50% of the asymmetric forms (~10% of 17S, ~25% of 13.3S, and ~15% of 9S).

Enzyme Assay (pH-Stat Method). In a total volume of 5 mL, the mixture contained 10^{-3} M acetylcholine iodide (AChI) or bromide and various concentrations of the chlorides of the salts of mono- and divalent metal ions. The reaction was started by adding 5 μL of enzyme solution (~1 IU).[1] To keep the pH constant 0.01 M NaOH was added by the use of a pH meter equipped with an impulsomat and a dosimat. The temperature was 25 °C, and the reaction mixture was kept under nitrogen. For activity determinations at varied salt concentrations the pH was kept at 7.4. If the activity at varied pH value was measured, the pH dependency of the pH-stat method had to be taken into consideration. At low pH (<6) the protonation of the acetic acid increases, thus leading to a reduced demand for NaOH. At high pH values (>9.5) the amount of NaOH is too large because of the increased deprotonation of the choline groups.

Results and Discussion

Effect of Monovalent Cations (Na^+ and K^+) on the Enzymatic Activity. Figure 1 shows the results of activity measurements at different sodium concentrations in the form log

† From the Laboratory for Physical Chemistry, Swiss Federal Institute of Technology, CH-8092 Zurich, Switzerland (P.H. and U.P.F.), and the Institute of Pharmacology, University of Zurich, CH-8006 Zurich, Switzerland (W.H.H.). *Received August 10, 1983; revised manuscript received November 23, 1983.* This work was supported by the Swiss National Science Foundation (Project 3.549-3.79) and by the Emil Barell Foundation (Hoffmann-La Roche, Project 127).

[1] Abbreviations: AChE, acetylcholinesterase (EC 3.1.1.7); AChI, acetylcholine iodide; 1 IU, 1 international unit = 1 μmol of product produced/min.

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